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Transgenic mice containing a human heavy chain immunoglobulin gene fragment cloned in a yeast artificial chromosome

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We have developed a method for the introduction of yeast artificial chromosomes (YACs) into transgenic mice. An 85 kilobase (kb) fragment of the human heavy chain immunoglobulin gene was cloned as a YAC, and embryonic stem cell lines carrying intact, integrated YACs were derived by co-lipofection of the YAC with an unlinked selectable marker. Chimaeric founder animals were produced by blastocyst injection, and offspring transgenic for the YAC were obtained. Analysis of serum from these offspring for human heavy chain antibody subunits demonstrated expression of the YAC-borne immunoglobulin gene fragment. Co-lipofection may prove to be a highly-successful means of producing transgenic mice containing large gene fragments in YACs.

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It has often been observed that cDNA based transgenes are poorly expressed or inappropriately regulated[1]. Genomic DNA-based transgenes which retain the content and organization of the locus are much more likely to be correctly expressed[2] but are limited by the cloning capacity of plasmid vectors. The yeast artificial chromosome (YAC) is a recently developed cloning vehicle with a capacity of over 2 megabases (Mb)[3]. The ability to introduce YACs into transgenic mice would significantly surpass current transgene size limits.

Several groups have successfully introduced YACs into fibroblasts[4], embryonal carcinoma cells[5] and CHO cells[6] by fusion of spheroplasts. Although endogenous yeast chromosomal DNA also integrated, the YACs were essentially intact and were expressed. Alternative transfection methods such as calcium phosphate precipitation[7] and lipofection[8] have resulted in successful transfer of YACs into mammalian cells. Recently, an embryonic stem (ES) cell line carrying a 320 kilobase (kb) YAC was produced by spheroplast fusion[9]. Because ES cells injected into blastocyst stage embryos can contribute to the germline of the resultant animal, YAC transfer into ES cells represents one route to transgenesis of YACs. An alternative approach, microinjection of zygotes, has been applied successfully to the production of transgenic mice carrying a small (35 kb) YAC encoding the tyrosinase gene[10]. The insert was cloned in a high copy number YAC vector[11] to facilitate YAC DNA recovery, and expression of tyrosinase was evident as coat colour pigmentation.

We are applying YAC transgenesis toward the production of transgenic mice carrying unrearranged human immunoglobulin genes. These mice could be used to derive hybridomas producing fully human monoclonal antibodies. Because they lack the intrinsic immunogenicity of non-human immunoglobulin molecules, human antibodies could lead to new and broad applications of immunoglobulin therapy.

In this study, we have cloned an 85 kb fragment of the human immunoglobulin heavy (H) chain gene containing at least one of each element required for correct rearrangement and expression of a human IgM molecule in a YAC. This YAC was introduced into ES cells by co-lipofection with an unlinked selectable marker plasmid. The co-lipofection strategy differs from lipofection of modified YACs in that retrofitting vectors do not need to be constructed or recombined into the YAC, and YACs carried in recombination-deficient hosts can be used. It is likely that larger YACs can be introduced by co-lipofection than microinjection due to the technical hurdles in purification of intact YAC DNA and because of the high shear forces imparted on the DNA during microinjection. Furthermore, unlike fusion of yeast spheroplasts with mammalian cells, no yeast chromosomal DNA is introduced in co-lipofection as the YAC is first isolated by pulsed field gel electrophoresis (PFGE).

We produced transgenic mice by blastocyst injection of ES cells carrying an intact YAC. The YAC was maintained intact through the germline, and H chain antibody subunits were detected in the serum of transgenic offspring.

Cloning of J1.3P

An 85 kb *Spe*I restriction fragment of the H chain gene contains V_H6, the functional diversity (D) segments, all six joining (J) segments, and the Cμ constant region segment[12–14]. A size selected (50–100 kb) *Spe*I complete digest YAC library was produced in pYACneo[15], and

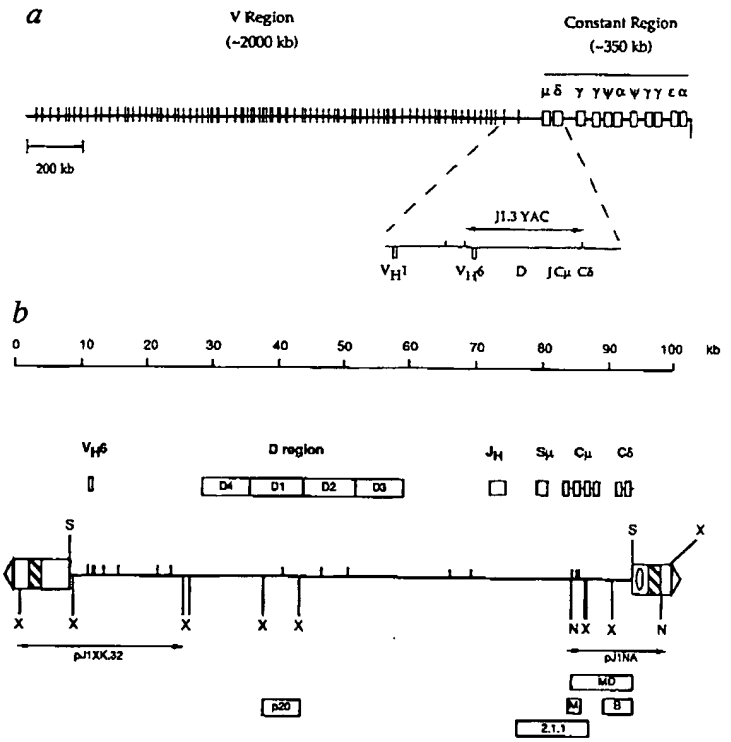

Fig. 1 *a*, The human immunoglobulin heavy chain locus. Approximately 150 V_H genes, denoted as vertical lines, are distributed over 2 Mb. The D and J regions are found within a 60 kb region, and the 11 constant region segments are distributed within a partially duplicated cluster of 350 kb. *b*, J1.3PYAC. The YAC vector arms are denoted as blocks flanking the human heavy chain DNA insert. The striped boxes in the left and right arms denote the URA3 and TRP1 genes respectively. The position of the V_H6 gene segment, the D region repeats, the J segment cluster, the Cμ switch region (Sμ) the Cμ exons, and the Cδ elements are shown above the restriction map. The triangles denote telomeres, and the circle in the right arm denotes the centromere. *Eco*RI sites are denoted as an unlabeled tickmarks, while S,X, and N denote *Spe*I, *Xho*I and *Nde*I sites, respectively. (Not all *Nde*I sites are shown.) The arrowed lines denote the fragments cloned by direct subcloning using *Xho*I partial digestion (pJ1XK.32) and *Nde*I digestion (pJ1NA). The boxes p20, 2.1.1, M, B, and MD denote hybridization probes for the D region (5.1 kb *Bam*HI fragment), Cμ region (12 kb *Xho*I fragment), Cμ region (0.9 kb and 1.2 kb *Eco*RI fragments), 3' end region (5.8 kb Bam HI-Spe I fragment) and 3' end region (10.5 kb Nde I-Spe I fragment) respectively.

screened by colony hybridization with a probe specific for human Cμ (probe 2.1.1, Fig. 1*b*). One positive clone (J1) was identified among approximately 18,000 primary transformants. Because yeast mitochondrial DNA often obscured the YAC on pulsed field gel electrophoresis, a ρ° petite variant lacking mitochondrial DNA was selected[16], and denoted J1.3P.

We subcloned the ends of the J1.3P insert using the bacterial selectable markers in the centromeric and acentromeric arms of pYACneo (Fig. 1*b*). Restriction analysis of the terminal fragments was consistent with published data[14,17], and defined the orientation of the insert with respect to the vector arms. The orientation was further verified by polymerase chain reaction (PCR) analysis of the acentromeric insert for V_H6 sequences, and hybridization of the centromeric insert with the Cμ probe 2.1.1 (not shown). Southern analysis of the Cμ region was consistent with published maps and restriction analyses[12] (not shown). The functional diversity segments of the H chain are contained in a 35 kb span containing a fourfold polymorphic repeat of D segments[18,19]. Southern analysis of the J1.3P YAC produced a "restriction fragment fingerprint" of the D region in which all of the D specific bands in the YAC were present in human genomic DNA (Fig. 2*b*).

Co-lipofection of J1.3P YAC into ES cells

The J1.3P YAC was co-lipofected with an unlinked linearized plasmid carrying the neo^r gene driven by the mouse phosphoglycerate kinase (PGK) promoter[20]. Two different plasmids were tested (see Methodology): pYPNN and pPGKneo, both carrying the same PGKneo cassette.

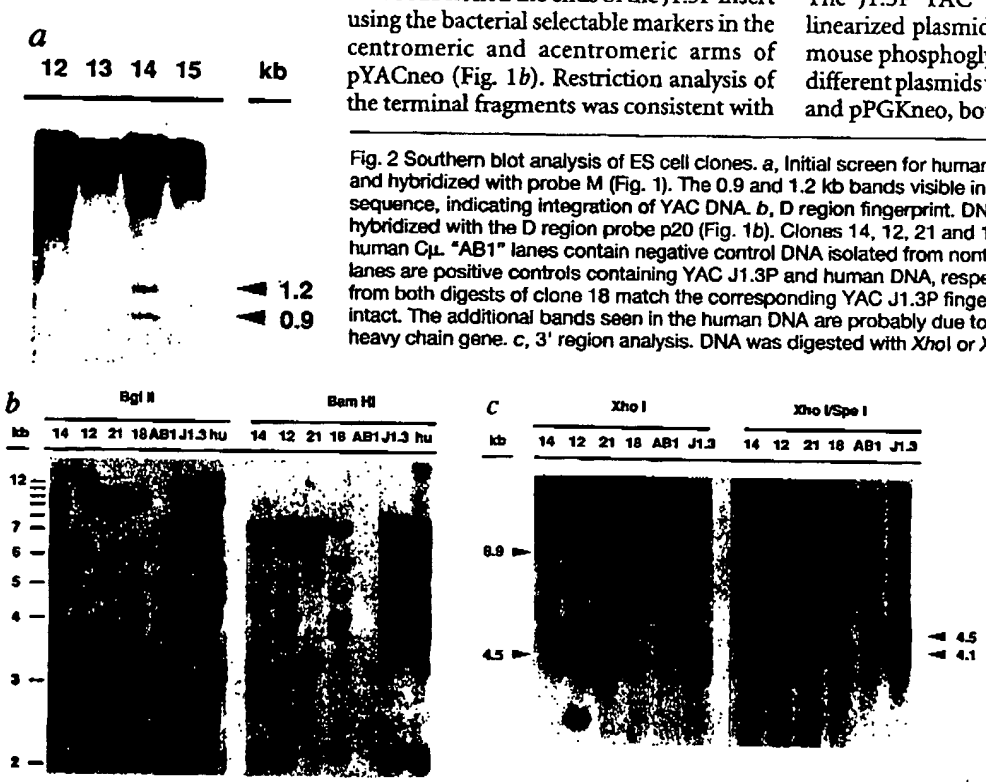

Fig. 2 Southern blot analysis of ES cell clones. *a*, Initial screen for human Cμ. ES cell DNA was digested with *Eco*RI and hybridized with probe M (Fig. 1). The 0.9 and 1.2 kb bands visible in clone 14 are diagnostic of human Cμ sequence, indicating integration of YAC DNA. *b*, D region fingerprint. DNA was digested with *Bgl*II or *Bam*HI and hybridized with the D region probe p20 (Fig. 1*b*). Clones 14, 12, 21 and 18 were positive in the initial screen for human Cμ. "AB1" lanes contain negative control DNA isolated from nontransfected ES cells. "J1.3", and "hu" lanes are positive controls containing YAC J1.3P and human DNA, respectively. The patterns of labelled bands from both digests of clone 18 match the corresponding YAC J1.3P fingerprints, indicating that the D region is intact. The additional bands seen in the human DNA are probably due to "orphon" D segments unlinked to the heavy chain gene. *c*, 3' region analysis. DNA was digested with *Xho*I or *Xho*I + *Spe*I and hybridized with probe MD (Fig. 1). The *Xho*I digest of J1.3P shows 2 diagnostic bands of 8.9 and 4.5 kb; a predicted third band (>30 kb) is not visible as it is larger than the shear limit of DNA fragments produced by the DNA isolation method used (~ 20 kb). ES clones 12, 21 and 18 have the diagnostic 4.5 kb *Xho*I band and clone 18 also has an 8.9 kb band that indicates retention of much of the vector arm on the 3' end. The *Xho*I-*Spe*I double digest reduced the 8.9 kb band to 4.1 kb in the YAC J1.3P control and clone 18 DNA, but clones 14, 12, and 21 have aberrant patterns; the faint 8.9 kb band in the *Xho*I-*Spe*I digest of clone 18 DNA is a *Spe*I partial digest artifact.

Table 1 Summary of J1.3PYAC Lipofections

Expt. No.	Selection plasmid	YAC: neo molar ratio	No. of lipofections	No. of G418^r clones	No. Cμ positive	Clone No.
1	pYPNN	1:8	8	24	3	14, 18, 21
2	pYPNN	1:8	9	19	1	12
3	pYPNN	1:8	9	12	0	
4	pYPNN	1:8	9	8	0	
5	pYPNN	1:8	9	10	0	
6	pPGKneo	1:4	4	44	0	
7	pPGKneo	1:4	16	480	3	86, 266, 480
8	pPGKneo	1:4	18	624	10	25,35,164, 191,195, 220, 371, 463, 553, 567

Approximately 100 ng of YAC J 1.3 was combined with the PGKneo-containing plasmids pYPNN or pPGKneo and carrier DNA. Each experiment consisted of 4 to 18 separate lipofections of 100 ng YAC DNA each using the same solution of DNA/lipid complexes. The human Cμ probe (M) was used for initial screening of clones. The two selectable marker plasmids, pYPNN and pPGKneo, produced frequencies of 0.5 and 13.5 G418 resistant clones per 10^6 transfected cells, respectively; the efficiency of pYPNN selection was much lower even though it was used at twice the molarity of pPGKneo. This may be due to the extent of sequence homology between the plasmid and the vector arms, or different efficiencies of neo^r expression.

The YAC:plasmid molar ratio was 1:8 for pYPNN and 1:4 for pPGKneo. Two cationic lipid formulations were tested, DOGS (Transfectam; Promega) and DOTMA (Lipofectin; BRL). Similar transfection efficiencies were obtained for each with linearized plasmids, but DOGS was chosen for the YAC experiments because its cationic moiety is spermine, obviating the need for exogenously added spermine as a DNA protectant[8], and because DOGS was not toxic to ES cells at the concentrations used (not shown). Because the DNA:lipid ratio is important for transfection efficiency, and precise measurement of the YAC DNA concentration was difficult, each lipofection contained an estimated ten-fold excess (1 μg) of sheared herring sperm carrier DNA to provide a baseline level of DNA.

Approximately 8 μg of J1.3P YAC DNA were lipofected in eight experiments (Table 1). Of the 1221 G418 resistant clones screened, 15 contained the two diagnostic *Eco*RI Cμ fragments (Fig. 2*a*; Table 2).

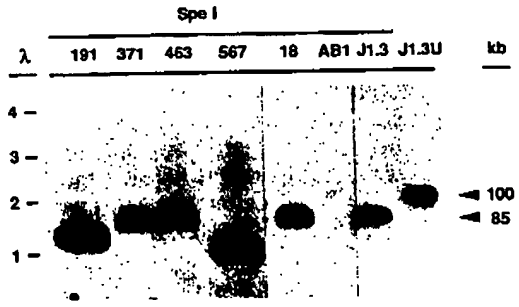

Fig. 3 Pulsed field analysis of ES cell clones. ES cells were embedded in agarose, digested with *Spe*I, and hybridized with probe p20 (D region). Clones 18, 371, and 567 have a single *Spe*I fragment of 85 kb, indicating integration of at least one intact YAC. Clones 191 and 567 have smaller fragments, indicating loss of one or both Spe I sites in the YAC, or internal loss of YAC sequences. Control lanes are as follows: AB1, ES cells; J1.3, parent YAC containing yeast strain; J1.3U, undigested parent YAC yeast strain. The arrows mark the position of the undigested YAC and the 85 kb *Spe*I fragment respectively. λ denotes lambda ladder marker.

Analysis of YAC structure in ES cells

The four Cμ$^+$ clones from the pYPNN co-lipofections (12,14,18,21) were analysed for D region structure by the fingerprint assay described above (Fig. 2*b*). Only clone 18 retained the fingerprint of the parent YAC. Clones 14 and 21 contained fewer bands than the parent, suggesting that YAC sequences may have been lost, while clone 12 contained several additional bands, consistent with integration of more than one copy of the YAC.

The integrity of the 3' end of the insert region in the four ES lines was assessed by Southern analysis using as probe the 10.5 kb *Nde*I–*Spe*I terminal fragment isolated by vector recircularization (Fig. 1*b*). Three bands are expected from a *Xho*I digest of the parent YAC: a very large D–J–Cμ band (>30 kb), a 4.5 kb Cμ-Cδ band, and an 8.9 kb Cδ–vector band. A double digest with *Xho*I and *Spe*I is expected to reduce the size of the 8.9 kb band to 4.1 kb. Indeed, J1.3 DNA gives the predicted pattern. Among the four ES lines, only line 18 contained the parental YAC banding pattern indicative of an intact 3'-end (Fig. 2*c*). The presence of an 8.9 kb band is consistent with the retention of the vector arm *Xho*I site, suggesting that very little of the telomeric region had been lost. A similar analysis of 5' end integrity was not possible due to repetitive elements in the region. However, PCR and Southern analysis using the V_H6 PCR product as probe indicated that clone 18 contained V_H6 sequences, while clones 14, 12, and 21 did not (Table 2).

Of the 13 Cμ$^+$ ES cell lines from the pPGKneo co-lipofections, one was lost during clonal expansion, and one (266) was eliminated because it lacked V_H6 sequence. The remainder were analysed for D region structure, 3'-end integrity and/or V_H6 sequence (Table 2). Six (86, 191, 220, 371, 463, 567) showed an intact D region while five had aberrant patterns. 3' end analysis of five of the six lines with intact D regions revealed that all but one (220) contained an intact 3' end. PCR analysis revealed that five of the six lines with intact D regions (86, 220, 371, 463, 567) contained V_H6 sequences, while only one of five lines without intact D regions (35) contained V_H6 sequences.

Ten of the ES cell lines were examined for full length insert by pulsed field Southern analysis using the D region or Cμ probe (Table 2). Only clones 18, 371 and 463 contained an 85 kb *Spe*I fragment indicative of a full length insert; all of the other clones had a smaller *Spe*I fragment (Fig. 3 and not shown). The *Spe*I digest of clone 18 was screened with D, Cμ and V_H6 probes; all three probes hybridized to a single band of 85 kb (not shown).

Taken together our data indicate that the YAC insert was transferred intact in three ES lines: 18, 371 and 463. A high degree of internal rearrangement, deletion or fragmentation was generally seen in the ES lines carrying disrupted YAC sequences, although subtle alterations of structure were also detected (for example, 567). Overall, the frequency of intact YAC transfer was low, 1 in 400 G418^r clones (3/1221). However, the isolation of the clone DNAs and the primary screen for Cμ sequences (which eliminated 1,206 of the 1,221 clones from further analysis) were rapidly performed using microtitre plate protocols (see Methodology). Thus, only 15 clones required extensive analysis (Table 2).

Molecular analysis of YAC structure in ES cells is greatly facilitated by a low, preferably single, copy of the YAC. The D region, PFGE and 3' end analyses of the ES lines are consistent with a low or single copy integration of the

Table 2 Summary of ES clone structural analysis

Clone No.	12	14	18	21	25	35	86	164	191	195	220	266	371	463	480	553	567
VH6 (PCR)	–[a]	–[a]	+	–[a]	–	+	+	–	nd	–	+	–	+	+	nd	–	+
D region	–	–	+	–	–	–	+	–	+	–	+	nd	+	+	nd	–	+
Cμ analysis	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+
3' end	–	–	+	–	+	+	nd	–	+	–	–	nd	+	+	nd	–	+
PFGE analysis	–	–	+	–	nd	nd	–	nd	–	nd	–	nd	+	+	nd	nd	–

ES clones which were positive for the Cμ probe were analysed for V_H6 sequences by PCR, D region integrity (Fig. 2b) by Southern blot with probe p20, and 3' end integrity (Fig. 2c) by Southern blot with probe MD. Probe locations are indicated in Fig. 1. + in the V_H6 analysis row denotes amplification of a diagnostic 275 bp PCR product using V_H6 specific primers. + in the D region row fingerprint denotes a completely parental banding pattern. + in the Cμ analysis row signifies that two diagnostic bands were present when hybridized with probe M, while – indicates the presence of a single band (Fig. 2a). + in the 3' end analysis row denotes the reduction in size of the flanking *Xho*I band to 4.1 kb in a *Xho*I/*Spe*I double digest, as well as the presence of a 4.5 *kb* internal *Xho*I band in both digests. + in the PFGE analysis denotes the presence of a single 85 kb *Spe*I band when probed with the D region probe p20. nd, not determined.
Two clones (195 and 553) contained only one of the two Cμ bands, which may have arisen from fragmentation of the YAC within the missing *Eco*RI fragment.
[a]Clones 12, 14, 18 and 21 were analysed for VH6 by probing *Apa*I and *Eco*RI digests using the 275 bp PCR product DNA as probe. Only clone 18 was positive for the diagnostic V_H6 bands.

YAC. Analysis of the intact clones for a diagnostic 3' end flanking band showed that clones 18 and 371 carried a single copy of the YAC insert, while 463 may have an additional intact or partially intact copy (Fig. 4). Strauss and Jaenisch[8] reported that lipofection into fibroblasts of a 150 kb YAC carrying an integral neo^r marker in the vector arm resulted in multiple partially deleted copies of the YAC in all G418^r clones, some of which also contained one or more intact YACs. The difference in copy number may be due to the different cell types (ES versus fibroblast) or to the species of cationic lipid employed. Alternatively, if the ends of the YAC are frequently degraded[21], multiple copies of YACs with integral neo^r markers may need to be integrated to permit the survival of at least one functional neo^r cassette.

Production of chimaeras and germline transmission

Blastocysts were injected with ES lines 18, 371 and 463 (ref. 22). Chimaeric founder animals ranging from 10% to 95% ES cell contribution to coat colour were derived from all three lines. The oldest animal, a 40% chimaeric male derived from ES line 18, transmitted the ES cell genotype to 20 of 73 offspring. Eleven of the 20 agouti offspring were positive for an intact D region fingerprint, consistent with Mendelian segregation of a hemizygous YAC transgene allele (Fig. 5). Southern analysis using the D region probe demonstrated a single 85 kb *Spe*I band in transgenic offspring, indicating that the YAC was stably maintained through the germline (not shown). Thus, co-lipofection of YACs into ES cells does not abrogate ES cell totipotency.

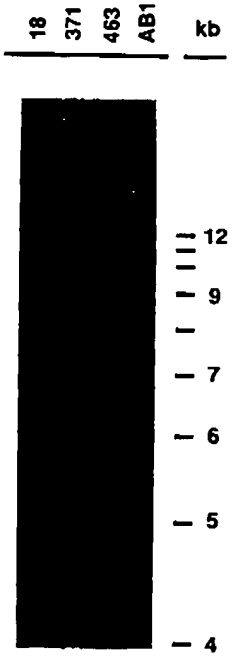

Fig. 4 Copy number of YAC J1.3P in ES cell clones. YAC-containing ES cell DNA and negative control DNA (AB1) was digested with *Bam*HI and hybridized with the probe B (Fig. 1*b*). Since the 3' vector arm lacks a *Bam*HI site, the size of the band is determined by the location of the flanking *Bam*HI site in the ES cell genome, and number of bands indicates the number of copies of the 3' region in the ES cells. Clones 18 and 371 each show a single band, consistent with single copy integration of the YAC, while clone 463 has two bands, indicating the presence of at least a partial second copy.

Southern analysis of integration sites for the co-lipofected selectable marker indicated integration of 2 to 10 plasmid copies (not shown). Because the marker plasmids could be a source of mutations, the integration sites of the plasmid were tracked by Southern analysis for plasmid sequences. Since pYPNN and the YAC vector arms lack *Eco*RI sites and contain pBR322 sequences, each *Eco*RI band which hybridized to a pBR322 probe would represent the integration of a separate intact or fragmented copy of pYPNN or vector arms. Analysis of ES cell clone 18 DNA revealed eight *Eco*RI bands ranging in size from 5.5–20 kb (not shown), and the offspring of a hemizygous transgenic animal bred with non-transgenic mates were analysed for segregation of the *Eco*RI bands. Among 14 offspring, all eight *Eco*RI bands were detected in tail DNAs of the nine transgenic pups, and none were detected in tail DNAs of the five non-transgenic pups (not shown). Thus, all detectable marker plasmids segregated with the YAC, indicating that they had inserted at or near the YAC integration site. Co-integration of different DNAs have been observed in transgenic mice produced by microinjection of zygotes[1], and co-integration of plasmid DNAs is probably no more mutagenic for co-lipofection than for microinjection. Presumably, the herring sperm carrier DNA had also co-integrated with the YAC, and may be a source of *Eco*RI sites in the Southern analysis. As co-integrated carrier DNA may adversely affect YAC transgene function, it would be preferable to omit carrier DNA. Preliminary experiments with a 650 kb YAC indicate that carrier DNA is not required for efficient lipofection of intact YACs into ES cells and that the size limit of YACs which can be co-lipofected into ES cells is at least 650 kb (T.K.C., unpublished observations).

Serum expression of human antibody subunits

Line 18 transgenic mice were assayed for human μ chain in the serum by ELISA. Human μ chain was detected in the serum of transgenic offspring (Table 3). Human μ serum levels were very low compared to serum levels of endogenous mouse IgM. The low level of transgene expression is due in part to competition from the endogenous H chain gene. When the transgene was

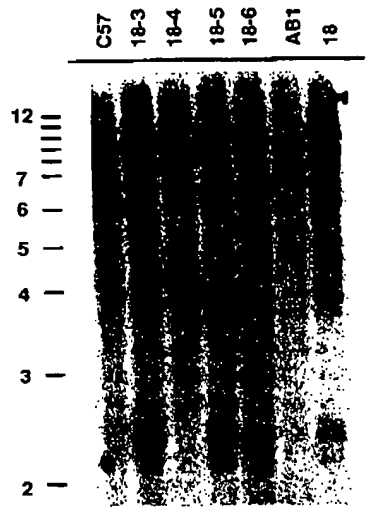

Fig. 5 Germline transmission of YAC J1.3. Tail DNAs from four agouti offspring of a clone 18 founder chimaera bred with C57BL/6 females were analysed for D region fingerprint (Fig. 2*b*). The C57 lane contains DNA from a nonagouti littermate, and AB1 and 18 lanes contain ES cell DNA from negative and positive control ES cell lines, respectively. Pups 18-3, 18-5 and 18-6 are transgenic for the YAC.

introduced into a background in which the endogenous heavy chain alleles are inactivated[23], the human μ serum levels were elevated approximately tenfold (Table 3).

Discussion

We have cloned a fragment of the human H chain gene as a YAC and introduced it intact into transgenic mice by co-lipofection of ES cells. The YAC was stably transmitted to offspring and directed the production of human IgM antibody subunits in the serum. Although the J1.3P YAC is small relative to the cloning capacity of YACs, our co-lipofection methods should be applicable to YACs of any size, effectively removing the size limitation on transgenes. Preliminary experiments indicate that YACs of at least 650 kb can be introduced intact into ES cells using co-lipofection (T.K.C., unpublished observations).

Co-lipofection

Co-lipofection offers several advantages over lipofection of modified YACs[8], microinjection of zygotes[10] and spheroplast fusion[9] for introducing YACs into mammalian cells and transgenic mice. First, unlike lipofection of modified YACs or spheroplast fusion, existing YACs can be used without modification. To allow for selection of YACs in mammalian cells, neomycin resistance (neo^r) selectable markers have been homologously recombined in yeast into the vector arms[9] or into alu repeat elements within the YAC insert[9,24]. Several plasmids have been developed to retrofit the vector arms of pre-existing YACs with a neo^r cassette[25]. By co-lipofecting YACs and unlinked selectable marker, several weeks of YAC engineering can be avoided. For instance, by co-lipofecting plasmids bearing an alternative selectable marker such as puromycin resistance[26], YACs can be introduced into cells that are already G418 resistant, such as ES lines carrying a targeted disruption, without the need to build novel retrofitting plasmids. In some cases, retrofitting a YAC may not be possible. For example, many human genomic fragments contain tandem repeat sequences which are unstable in yeast[27], and one approach toward cloning of unstable sequences is to construct the YAC library in a *Rad52* deficient host[27,28]. The *Rad52* mutation stabilizes YACs by blocking recombination, but also precludes recombinational modification of YACs. Furthermore, strategies which place a selectable marker within the insert sequences[24] may disrupt the transgene or interfere with its expression, while those which place the marker into YAC vector arms require that the YAC ends survive transfer for selection. Since 90% of mammalian cell clones carrying YACs suffer loss of terminal sequences[5,6,21], a large fraction of clones carrying YACs may be missed when the selectable marker is located in the YAC arm. Co-lipofection does not require that the YAC ends survive transfer for successful recovery of the YAC containing clone.

Second, unlike microinjection approaches, purification of the YAC is not required. For co-lipofection, the YAC is separated by PFGE, the gel matrix is digested with agarase, and directly mixed with the cationic lipid for application to cells. In contrast, DNA for microinjection must be extensively purified, increasing the risk of damage by shearing. Furthermore, microinjection of zygotes requires precise quantification of the DNA solution which may be difficult due to the low concentration of YAC DNA in the pulsed field gel. Schedl *et al.*[10] were able to purify and microinject a 35 kb YAC using YAC amplification and ethanol purification. But although effective for small YACs, ethanol purification may not be suitable for large YACs[29].

Third, pulsed field gel isolation of the YAC for co-lipofection precludes the transfer of yeast chromosomal DNA, unless the YAC is by chance the same size as a yeast chromosome. In contrast, spheroplast fusion methods introduce approximately half of the yeast chromosomes along with the YAC into target mammalian cells[5,6].

The main disadvantage of co-lipofection is that only a fraction of the selected clones contain the YAC DNA.

Table 3 Detection of serum human μ chain by ELISA

Genotype	Sex	Age at assay (wk)	Human μ
YAC 18+	F	3, 9, 20	< 5 ng ml^{-1}
YAC 18+	M	17	12.2 ng ml^{-1}
YAC 18+	F	10	27.0 ng ml^{-1}
YAC 18+	F	6, 17	< 5 ng ml^{-1}
YAC 18+	F	4	5.8 ng ml^{-1}
YAC 18+	F	6	10.5 ng ml^{-1}
YAC 18+	M	6	10.4 ng ml^{-1}
YAC 18+/J_H^-	M	5, 8	165 ng ml^{-1}[a]
Wild type	F	6	< 5 ng ml^{-1}
Wild type	F	6	< 5 ng ml^{-1}
Wild type	F	6	< 5 ng ml^{-1}
Wild type	M	6	< 5 ng ml^{-1}
Wild type	F	34	< 5 ng ml^{-1}
Wild type	F	34	< 5 ng ml^{-1}
Wild type	M	34	< 5 ng ml^{-1}
Wild type	M	34	< 5 ng ml^{-1}

Blood samples were analysed by ELISA for human IgM at the ages indicated. All of the transgenic animals are derived from a single clone 18 founder chimaera, and are hemizygous for the YAC (YAC 18+). Five of the seven animals in a wild type background had detectable human IgM in their serum. The level of detection of the ELISA was 5 ng human μ serum (see Methodology). The serum human μ level was elevated approximately tenfold when the YAC transgene was bred into a background lacking functional endogenous mouse heavy chain genes[23] (YAC18+/J_H^-).
[a]Mean of two measurements of 183 ng ml^{-1} and 146 ng ml^{-1}.

However, because it is not uncommon to find frequencies of 1 in 400 or less in gene targeting experiments with ES cells, protocols for simplified screening of large numbers of ES cell clones have been developed. Using microtitre plates for all manipulations of ES cell clones, analysis of many hundred clones takes only a few days[30].

Expression of J1.3 in mice

Many factors might affect transgene expression and account for the low level of serum human μ chains in line 18 transgenic mice, with or without competition from mouse H chain gene expression. Expression of the transgene may be influenced by neighbouring sequences (position effect). Flanking plasmid, carrier and vector DNAs may also adversely affect expression, as plasmid sequences often suppress expression in transgenics generated by microinjection[1]. Presumably, larger YAC transgenes containing longer flanking sequences would "buffer" the transgene from plasmid or position effects.

Alternatively, some fraction of transcripts may be spliced to produce a nonfunctional protein. Post-transcriptional processing of the IgM/IgD primary transcript is complex and highly regulated[31]. The Cδ portion of the IgD molecule is encoded by alternative splicing among eight Cδ exons (Cδ1, CδH1, CδH2, Cδ2, Cδ3, CδS, CδM1, CδM2) and, by their omission, the six Cμ exons. Because the *SpeI* site defining the 3' end of the transgene occurs between CδH1 and CδH2, transcripts for both the secreted and membrane bound forms of human IgM can be produced from the primary transgene transcript, whereas splicing from the VDJ exon into a Cδ exon would produce a truncated and presumably nonfunctional IgD molecule. Because the relative levels of IgM and IgD at different B cell developmental stages are regulated at the post-transcriptional level, a significant portion of transgene transcripts may be nonproductively spliced.

Another possible reason for the low expression levels is that the human antibody repertoire encoded by the single V_H6 segment contained in the J1.3 YAC transgene may not efficiently substitute for mouse heavy chains during mouse B cell development. V_H6 antibodies are typically found in the human fetal rather than the adult repertoire[32], and V_H6 to DJ joining, V_H6 expression, or development of V_H6 antibody expressing B cells may be inefficient in mature mice. Alternatively, human antibodies in general may not efficiently participate in mouse B cell development.

Production of human antibodies in transgenic mice

There are several strategies for producing human monoclonal antibodies. Human B cells isolated from donors could be used to produce hybridomas, but tolerance to human antigens would limit the range of antibodies obtainable by this approach. Immunization of SCID mice engrafted with human immune tissue[33] could circumvent the tolerance limitation, but would still require production of human hybridomas which are more difficult to produce and maintain than mouse hybridomas. Human heavy and light chain variable regions could be randomly combined, expressed on the surface of bacteriophage, and screened for desired specificities to mimic affinity maturation *in vitro*[34]. This approach is limited by the requirement for purified antigen which is both stable and in its native conformation under phage screening conditions. In contrast, transgenic mice carrying unrearranged human immunoglobulin genes would permit derivation of human antibodies against all non-mouse antigens using the traditional hybridoma production methods developed for mouse B cells. The main drawbacks to this approach are that the transgenes must carry the sequences necessary to encode a complete repertoire, and that the human antibodies must successfully participate in mouse B cell development.

The gene fragment contained on the J1.3 YAC encodes only a limited repertoire of H chain antibodies. A broader diversity of human antibodies would require many V_H, D and J gene segments. Furthermore, because the highest affinity antibodies are produced by affinity maturation of IgG antibodies, the transgene must contain the necessary sequences for isotype switching and somatic mutation. The molecular genetic tools which are available for the recombination and modification of sequences in yeast offer a means to construct YAC H transgenes with additional V_H and constant region genes. Introduction of such heavy chain transgenes into mice that express analogous human light chain transgenes in a background lacking functional mouse immunoglobulin genes would permit analysis of human antibody functionality in mouse B cell development. Characterization of human immunoglobulin gene rearrangement, class switching, and affinity maturation in such mice may provide insight into these processes, and permit the derivation of mouse hybridomas expressing high affinity, fully human antibodies.

Note added in proof. While this report was in the press, three other groups[40,41,42] reported production of transgenic mice containing genes cloned in YACs.

Methodology

Cloning the human heavy chain gene. Fresh human sperm was harvested and genomic DNA prepared in agarose blocks as described[35]. A size selected (50–100 kb) *SpeI* complete digest YAC library was prepared in the yeast host strain AB1380 in pYACneo[15], using the *SpeI* site near the centromere as the cloning site. Clones containing human Cμ sequences were identified by colony hybridization[15]. One clone, J1, contained an 85 kb *SpeI* insert. Because yeast mitochondrial DNA often obscured the J1 YAC on pulsed field gels, subclones of J1 lacking mitochondrial DNAs were generated by ethidium bromide ρ^0 selection[16]. One subclone, J1.3P, was mounted in agarose blocks at 3.5×10^9 cells ml^{-1} and intact yeast chromosomal DNA prepared[11]. The YAC DNA was isolated in a 3–4 mm wide gel slice from a low melting point preparative CHEF gel (Biorad). The gel slice was equilibrated in β-agarase buffer (Gelase, Epicentre Technologies), melted at 70 °C for 20 min, cooled to 45 °C, and digested with 10 U of agarase overnight at 45 °C.

Selectable marker plasmids. pPGKneo is a 5 kb plasmid containing an expression cassette consisting of the neo gene under the transcriptional control of the mouse PGK-1 promoter and the PGK-1 poly (A) site[36]. pYPNN is a variant of pYACneo containing the PGKneo cassette in place of the SV40 promoter-neo^r cassette, constructed by exchange of a 4.5kb *SalI–ApaI* fragment of pYACneo for a 1.5 kb Sal I-Apa I fragment of pPGKneo containing the PGK promotor, neo^r coding region, and the PGKp(A) signal. The plasmids were linearized with *SalI* (pPGKneo) or *NotI* (pYPNN).

Lipofection of YAC DNA into ES cells. The digested agarose/DNA mixture was divided into 1 ml (about 100 ng) portions in polystyrene tubes (Falcon), 100 ng pYPNN or 20 ng pPGKneo, and 1 μg sheared herring sperm DNA (Sigma) was mixed in each tube, and cationic lipid (Transfectam, ProMega) added at a 10:1 ratio (wt:wt) and gently mixed into the DNA solution. The mixture was incubated for 30 min at room temperature to allow formation of DNA-lipid complexes. Rapidly growing confluent cultures of AB-1 ES cells on mitotically inactivated SNL 76/7 fibroblast feeder layers[37] were trypsinized to

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yield a single cell suspension, washed with serum-containing medium, and resuspended in serum-free DMEM (Gibco). For each lipofection, 9 ml of cell suspension containing 3×10^6 ES cells and about 1×10^5 feeder cells were mixed with 1 ml of the DNA-lipid mixture in a 60 mm petri dish (Falcon 1007; Becton Dickinson) and incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. Dishes were swirled gently during the incubation to minimize cell attachment. After incubation, cells were diluted with serum-containing ES cell medium[30], dispersed gently, and plated at 1×10^6 on 100 mm culture dishes containing feeder layers. Cells were selected in G418 (400 μg ml^{-1} powder, Gibco) for 9–12 days, beginning 24 h after plating.

Analysis of ES clones. G418-resistant clones were dispersed with trypsin and the cells from each clone were divided into one well of a 96-well plate that was frozen[30] and a second 96-well or 24-well plate used for preparation of DNA for screening by Southern analysis. Positive clones were thawed and expanded for further analysis.

Southern blot hybridization and PCR. Genomic DNA was prepared from ES cells and tail biopsies by rapid preparation methods[38,39] and subjected to Southern analysis by standard methods. For pulsed field gel electrophoresis, ES cells were embedded in agarose blocks at 10^7 cells ml^{-1}, prepared for restriction digestion, and digested overnight with *Spe*I. For Southern analysis of pulsed field gels, the DNA was acid-nicked, then transferred to GeneScreen Plus (DuPont) in denaturing solution (0.4 N NaOH, 1.5 M NaCl). Oligonucleotides suitable for PCR amplification of the V_H6 region were prepared from published sequences. Primers used were 5'CAGGTACA-GCTGCAGCAGTCA3' and 5'TCCGGAGTCACAGAGTTCAGC3', which amplified a diagnostic 275 bp product.

Production and analysis of transgenic mice. Clones containing intact YAC sequences were injected C57BL/6 into blastocysts to produce chimaeric founder animals[22]. These mice were bred with C57BL/6 wild type mice and J_H^- mice, which carry targeted inactivations of both copies of the mouse heavy chain gene[23]. Thymic cells from transgenic offspring were mounted in agarose blocks for pulsed field gel electrophoresis and Southern analysis to confirm transmission of the intact YAC.

ELISA assays. Human μ chain was detected using a 2-site ELISA assay. Polyvinyl chloride microtitre plates were coated with mouse monoclonal anti-human IgM clone CH6 (The Binding Site, San Diego, CA) at 1.25 μg ml^{-1} in 100 μl PBS by overnight incubation at 4 °C. Plates were blocked by 1 h incubation with 5% chicken serum (JRH, Lexana, KA) in PBS. Following 6 washes with PBS, 0.5% tween-20, serum samples and standards were diluted in 100 μl PBS, 0.5% tween-20, 5% chicken serum (PTCS) and incubated in the wells for 1 h at room temperature. Purified human myeloma-derived IgM, kappa (Calbiochem) was used as a standard. Plates were then washed 6 times with PBS, 0.5% tween-20 before addition of peroxidase conjugated rabbit anti-human IgM, Fc5u fragment specific antibody diluted 1/1000 in 100 μl PTCS. After another 1 h incubation at room temperature, the wells were washed 6 times and developed for 1/2 h with 100 μl ABTS substrate (Sigma). Assay plates were read at 415–490 nm on a Vmax microplate reader (Molecular Devices, Menlo Park, CA), and IgM concentration determined from a 4-parameter logistic curve fit of the standard values. A level of 4.89 ng ml^{-1} in serum samples is routinely detected by this assay and differentiated from background by at least 3 standard deviations.

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